CAROTENOIDS AND CHLOROPHYLLIC PIGMENTS IN THE MARINE SNAIL, CERITHIDEA CALIFORNICA HALDEMAN, INTERMEDIATE HOST FOR SEVERAL AVIAN TREMATODES [1, 2]

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The marine snail, *Cerithidea californica* Haldeman, is a favorable host for more than twenty species of larval trematodes (Martin, 1955; Hunter, 1942). These larvae occupy different regions of the body of the snail such as the digestive gland, mantle, gills and part of the digestive tract. The digestive gland of the snail, which is the main organ of infection, presents a variety of coloration in different specimens. It may be green, brown, yellow, orange or creamy white. The visceral part including the digestive tract is frequently dark blue. The mantle and the integument are usually greenish-blue or blue and yellow intermingled. A striking similarity exists between the coloration of the snail tissues and that of the parasitic larvae harbored by them.

A considerable amount of information is available concerning the occurrence and distribution of pigments, particularly carotenoids in various species of gastropod molluscs. Earlier work has been reviewed by Fox (1953) and Goodwin (1954). Although several species of snails are known to be hosts for pigmented larval trematodes, no critical study so far has been made concerning their pigments with a view to understanding the host-parasite relationship of pigmentation. In the snail, *Littorina littorea,* pigmented foot has been reported to be a means of recognizing infection with larval trematodes (Willey and Gross, 1957). Spectrophotometric absorption studies of *L. littorea* extracts indicated the presence of carotenoids; however, chromatographic methods were not used for the separation of various pigments. The author was interested to study the chemical nature and origin of pigments found in certain species of larval trematodes harbored by the snail, *Cerithidea* (Nadakal, 1960a, 1960b). In order to trace the host-parasite relationship of pigments, it was necessary to analyze the pigments of the snail. The present paper describes the pigments found in *Cerithidea* with special reference to carotenoids.

MATERIALS AND METHODS

Specimens of *Cerithidea* and four species of algae, including three green and one red algae which serve as food for the snails, were collected from the mud flats of Newport Bay, California. The green algae were identified as *Ulva* sp., *Chaeto-*

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morpha torta, and *Enteromorpha clathrata;* the red alga as *Hypnea johnstonii.* The algal pigments were studied by the methods of the following workers: Strain (1942) for chlorophyll *a* and *b;* Manning and Strain (1943) for chlorophyll *d;* and Haxo *et al.* (1955) for phycobilins. After sorting out the various snail tissues such as the digestive gland, mantle and foot, and visceral mass, they were lyophilized separately and ground in an ordinary mortar. The methods outlined by Fox and Pantin (1941) were followed with necessary modifications for the extraction and analysis of pigments from the snail tissues. Chromatographic separation of pigments was carried out according to the directions given by Karrer and Jucker (1950). A cylindrical glass tube measuring 20 cm. × 12 mm. was used for preparing the adsorption column. Among several adsorbents tried for the separation of various pigments, such as calcium hydroxide for epiphasic carotenoids, calcium carbonate and zinc carbonate for hypophasic carotenoids, and powdered sugar (C & H Confectioner's) for chlorophyll derivatives, activated alumina was found most satisfactory. Various pigment fractions obtained by chromatographic separation were eluted in appropriate solvents like petroleum-ether, methanol, etc. for determining the absorption spectra, with a Beckman Spectrophotometer. Efforts were made to identify the pigments by spectrophotometric absorption analyses, partition tests, color reactions, solubility, fluorescence, and chromatographic behavior.

Figures 1–3 include spectral curves of the pigments A-E in petroleum-ether (b.p. 50–70° C.) and F and G in methanol.

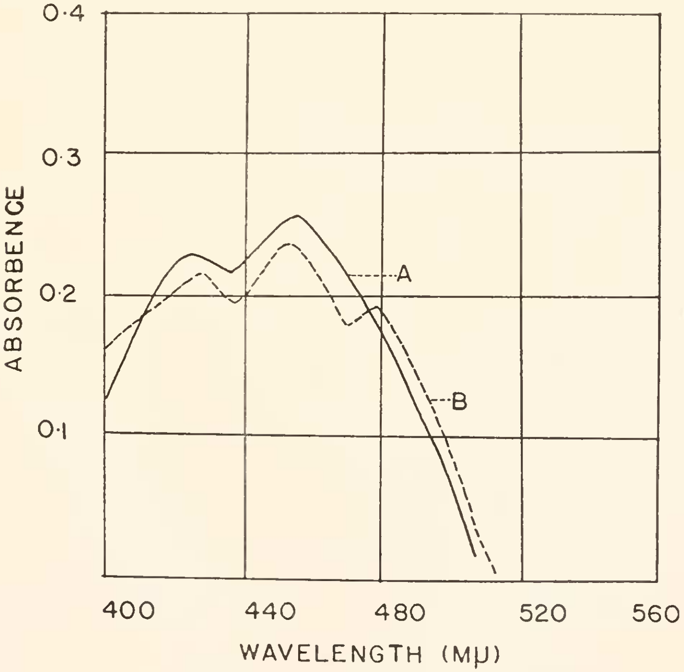

FIGURE 1. A. Light orange pigment HIII (Table II). B. Yellow pigment HII (Table II).

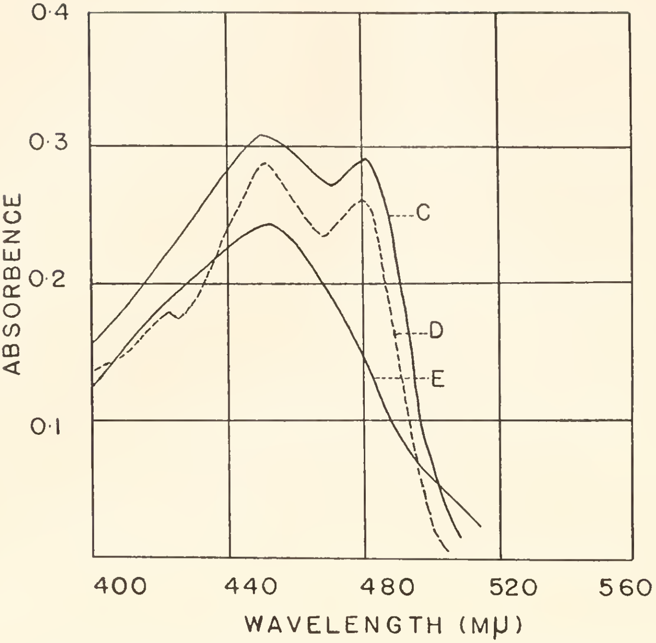

FIGURE 2. C. Orange pigment EIV (Table I). D. Orange pigment EIII (Table III). E. Violet pigment EIII (Table I).

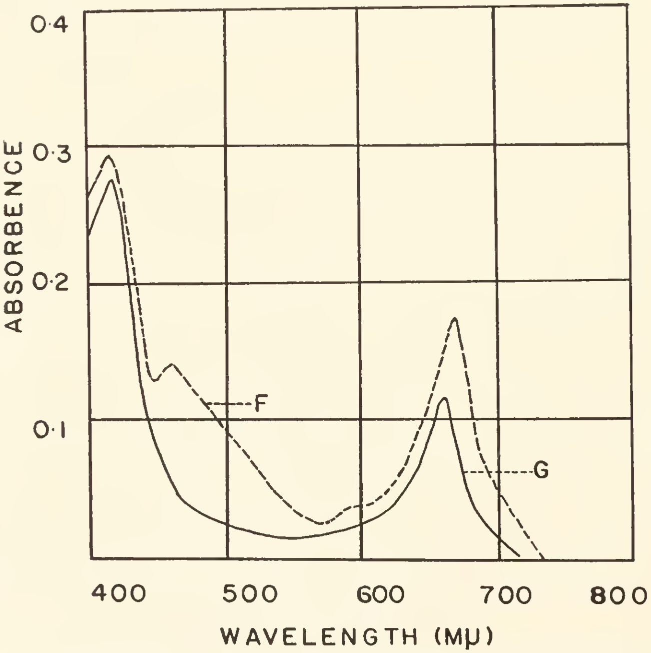

FIGURE 3. F. Yellowish-brown pigment EII (Table I). G. Pale green pigment EIIa (Table I).

RESULTS

The pigment fractions obtained by chromatographic separation of the epiphasic and hypophasic portions of the pigment extracts of the various snail tissues and their characteristics are listed in Tables I-V. The pigment fractions in the case of epiphasic portions of the extracts are numbered in order of decreasing adsorption and hypophasic portions in order of increasing adsorption on the columns.

Absorption maxima and the forms of the spectral curve (Fig. 3, G) of the pigments EI and EIIa (Table I) indicate that these pigments resemble chlorophyll or pigments derived from chlorophyll. However, the absorption maxima in the violet region of the spectrum are different from that of the chlorophyll reported from plant sources. The Gmelin reaction (Pearse, 1953) was negative for these pigments, suggesting that they are not open-ring tetrapyrrole compounds.

The brown pigment EIIb (Table I) showed a maximum at 450 mμ. This fraction could not be made hypophasic even after prolonged saponification. This may be a carotenoid acid.

TABLE I

Epiphasic portion of the digestive gland extract. Adsorbent: activated alumina. Developing solvent: petroleum ether (50–70° C.) with 1–5% methanol

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
EI	Greenish-yellow	2–3 methanol	410, 667	Ethanol
EII	Yellowish-brown	2–3 methanol	410, 450, 667	Ethanol
EIII	Violet	3–4 methanol	454	Petroleum ether
EIV	Orange	0	452, 482	Petroleum ether

Band EII was chromatographed again on alumina column and the two fractions obtained are given below:

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
EIIa	Pale green	2–3 methanol	416, 665	Methanol
EIIb	Brown	3–4 methanol with a few drops of glacial acetic acid	450	Petroleum ether

The violet pigment EIII (Table I) showed a maximum at 454 mμ. The form of the spectral curve (Fig. 2, E) and the single absorption maximum are suggestive of a keto-carotenoid (Vevers and Millott, 1957).

The orange pigment EIV (Table I) has been found to possess properties similar to those of β-carotene (Fig. 2, C). The absorption maxima are in good agreement with the figures given by Karrer and Jucker (1950), and Lederer (1938). Besides, solubility, behavior on partition test, fluorescence (bluish-green in ultra violet light), color reactions (pigment in chloroform solution turned bluish-green on addition of concentrated sulfuric acid), color in solutions, and chromatographic behavior also indicate that this is β-carotene.

The absorption maxima of the pink pigment HI (Table II) in petroleum ether and benzene are in good agreement with the figures given by Karrer and Jucker (1950), and Goodwin (1953) for zeaxanthin. Its hypophasic behavior on partition test and chromatographic behavior also lend support to the conclusion that

TABLE II

Hypophasic portion of the digestive gland extract. Adsorbent: activated alumina. Developing solvent: petroleum ether with 1–5% methanol

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
HI	Pink	2–3 ethanol	420, 450, 482	Petroleum ether
			462, 490	Benzene
HII	Yellow	1–2 methanol	426, 448, 478	Petroleum ether
			429, 457, 487	Chloroform
HIII	Light orange	1–2 methanol with a few drops of glacial acetic acid	424, 454	Petroleum ether
HIV	Blue-green	2–3 ethanol	416, 670	Methanol

this is zeaxanthin. The fact that this pigment fraction stayed hypophasic even after continued saponification and could not be made epiphasic with acid treatment, showed that this pigment occurs in the snail tissues in the free state.

The yellow pigment HII (Table II) showed maxima in petroleum ether and chloroform which are in good agreement with the figures quoted by Karrer and Jucker (1950) for the xanthophyllic pigment lutein (Fig. 1, B). This pigment turned bluish-green with concentrated sulfuric acid. These properties, coupled with the chromatographic behavior, hypophasic nature on partition test, and color in solutions, prove that this is lutein.

A yellow pigment fraction, separated from the epiphasic portion of the unsaponified pigment extract, showed maxima in petroleum ether at 424, 445, and 475 mμ. This pigment became hypophasic on saponification and could be made epiphasic again with acetic acid treatment. This indicated that some of the lutein in the snail's tissues is esterified. After saponification the pigment showed the maxima at 446 and 478 mμ in petroleum ether.

The light orange pigment HIII (Table II) could be removed from the column with a few drops of acetic acid in the eluting solvent. It is difficult to identify this pigment conclusively; it may be a carotenoid acid or some pigment derived from hypophasic carotenoids (Fig. 1, A).

The blue-green pigment HIV (Table II) is characterized by absorption maxima and spectral curve suggestive of chlorophyll or a pigment derived from it.

The brown pigment EI (Table III) showed only one absorption maximum.

TABLE III

Pigment extracts from the mantle, branchial, and pedal tissues. No hypophasic fraction was obtained on partition of the original extracts between 90% methanol and petroleum ether systems. Pigments chromatographed as the epiphasic portion of the digestive gland extract

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
EI	Brown	2–3 methanol	450	Petroleum ether
EII	Pink	1–2 methanol with a few drops of glacial acetic acid	452	Petroleum ether
EIII	Orange	0	424, 452, 480	Petroleum ether

This fraction could not be made hypophasic even after prolonged saponification in 90% methanol-petroleum ether systems. Identification of this pigment fraction was difficult.

The pink pigment EII (Table III) could be eluted with a few drops of glacial acetic acid. It showed a maximum at 452 mμ. On saponification this became hypophasic and could be made epiphasic again by treating with acetic acid. This behavior indicated that this pigment could be an esterified carotenoid acid.

The orange pigment EIII (Table III) showed all characteristics of β-carotene. However, a small shoulder at 424 mμ in the spectral curve of this pigment is remarkable (Fig. 2, D).

The khaki pigment EI (Table IV) showed an absorption maximum only in the violet region of the visible spectrum. This may be some breakdown product of chlorophyll or carotenoid pigments. Such breakdown products are known to be adsorbed at the top of the columns (Fox, 1953).

TABLE IV

Pigment extract from the visceral mass. Epiphasic portion chromatographed on activated alumina. Developing solvent: petroleum ether with 1–5% methanol

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
EI	Khaki	1–2 methanol	425	Methanol
EII	Greenish-yellow	2–5 methanol	416, 667	Methanol
EIII	Yellowish-brown	2–4 methanol	420, 452, 667	Methanol
EIV	Violet	2–3 methanol	452	Petroleum ether
EV	Orange	0	450, 482	Petroleum ether

Band EIII was chromatographed again on alumina column and the two fractions obtained are given below:

EIIIa	Pale green	1–2 methanol	416, 665	Methanol
EIIIb	Pale orange	1–2 methanol with a few drops of glacial acetic acid	450	Petroleum ether

The spectral properties of the pigments EII and EIIIa (Table IV) indicated that they are chlorophyll derivatives.

The pale orange pigment EIIIb (Table IV) is considered to be a carotenoid acid because of its single absorption band and acidic properties.

The violet pigment EIV (Table IV) was more or less similar to the violet pigment EIII (Table I) extracted from the digestive gland and may be a keto-carotenoid.

The orange pigment EV (Table IV) was identical with the pigments EIV (Table I) and EIII (Table III) extracted from the digestive gland and mantle, respectively. It is therefore concluded to be β-carotene.

The pink pigment HI (Table V) was similar in properties to the one HI (Table II) recovered from the hypophasic portion of the digestive gland extract and is concluded to be zeaxanthin.

The yellow pigment HII (Table V) has been identified as lutein, as its properties resemble those of the yellow pigment HII (Table II) extracted from the hypophasic portion of the digestive gland extract.

The light orange pigment HIII (Table V) with its acidic properties and two absorption maxima in the blue-violet region of the visible spectrum may be considered as a carotenoid acid. No trace of chlorophyll derivatives could be detected in the hypophasic portion of the visceral extracts.

A blue-green residue was left in the methanol-water fraction of the original extracts of the digestive gland and visceral tissues. Part of the colored substance could be taken up in ether after addition of a few drops of glacial acetic acid. It was then washed with water, evaporated to dryness under vacuum and finally taken up in methanol. The absorption maxima of this pigment at 416 and 667 mμ and the form of the spectral curve was characteristic of chlorophyll *a* (Atkins and Jenkins, 1953; Green, 1957). Even after extraction of the chlorophyllic pigments by acidified ether, a bluish residue was left behind in the aqueous methanolic solution. This was not extractable by any of the solvents tried. Attempts to separate the pigment on adsorption columns also failed. This bluish residue probably contained haemocyanin which is common in molluscan body fluids.

TABLE V

Hypophasic portion of the visceral extract. Chromatographed as the hypophasic portion of the digestive gland extract

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
HI	Pink	2–3 methanol	420, 450, 482	Petroleum ether
HII	Yellow	1–2 methanol	422, 448, 478	Petroleum ether
HIII	Light orange	1–3 methanol with a few drops of glacial acetic acid	424, 454	Petroleum ether

PIGMENTS FOUND IN THE ALGAE

The three species of green algae, *Ulva* sp., *Enteromorpha clathrata*, and *Chaetomorpha torta*, have been found to contain chlorophyll *a* and *b*, β-carotene, and the xanthophyllic pigment lutein. No trace of α-carotene or any other pigments related to carotenoids could be detected. The red alga, *Hypnea johnstonii*, contained chlorophyll *a* and *d*, β-carotene, lutein, phycoerythrin, and phycocyanin. The principal carotenoid pigment extracted from the algae was β-carotene.

DISCUSSION

The occurrence of carotenoids and chlorophyll derivatives in *Cerithidea* is in agreement with the previous findings in various species of molluscs. β-Carotene and lutein have been reported from several gastropods (Fox, 1953; Goodwin, 1954). Zeaxanthin is known to occur in *Patella vulgata* and *P. depressa* (Goodwin, 1954; Goodwin and Taha, 1950), and *Mytilus californianus* (Scheer, 1940). Carotenoid acids with single absorption bands in the visible spectrum have also been reported from many invertebrates such as sponges and molluscs (Fox, 1953). Demonstration of chlorophyll derivatives in molluscs has been made by MacMunn (1886a, 1886b), Dhéré and Vegezzi (1916), and many others.

A survey of the occurrence and distribution of carotenoid pigments in invertebrates reveals the fact that the digestive gland plays an important role in the storage of these pigments. Crane (1949) found that in the cephalopods, *Octopus bimaculatus* and *Loligo opalescens,* the liver-pancreas accumulated relatively large amounts of carotenoid pigments. In *Cerithidea,* undoubtedly the digestive gland functions as the chief organ for the storage of carotenoids. The preferential accumulation of carotenoids in the lipid-rich digestive gland is not surprising since these pigments have a tendency to be associated with lipids.

The visceral extracts of *Cerithidea* have also been found rich in carotenoid and other pigments. This might be due to the presence in the gut of plant materials ingested as food. The mantle and branchial tissues contained relatively small amounts of carotenoids and no traces of chlorophyll derivatives could be detected in these tissues. A similar situation is described by Brooks and Paulais (1939) in the lamellibranchs, *Ostrea edulis* and *Gryphaea angulata.*

In *Cerithidea,* the principal pigment found in various tissues is β-carotene. Next in importance, on a quantitative basis, are the chlorophyll derivatives; third comes the lutein and fourth only the zeaxanthin. Carotenoid acids and keto-carotenoids occur in traces only. The snail apparently shows a preference for storing β-carotene in its tissues. This is perhaps due to the preponderance of β-carotene in the algae which serve as food for the snail. Since *Cerithidea* assimilates and stores both hydrocarbons and xanthophylls as well as chlorophyll derivatives, it may be regarded as non-selective in its chromatic storage. However, the preferential accumulation of carotenes in the mantle and the branchial tissues is remarkable indeed.

There is ample evidence for the elementary origin of carotenoids and chlorophyll derivatives in animals. The crustacean, *Daphnia,* builds up its carotenoid supply from the algae upon which it feeds (Green, 1957). The sea mussel, *Mytilus californianus,* is known to absorb and store carotenoid pigments "from a very plentiful and widely varied diet" (Fox and Coe, 1943). Dhéré and Vegazzi (1916) concluded from experimental evidence that the greenish and grayish hepatic pigments of *Helix pomatia* were derived from the chlorophyll of its diet. The dark green pigment "chaetopterin" found in the intestinal epithelium of the polychaete worm, *Chaetopterus,* is derived from chlorophyll by the elimination of magnesium and the phytol chain (Lederer, 1940). Similarly, Lederer and Huttrer (1942) and Winkler (1957) showed that the sea slug, *Aplysia,* accumulates the pigment "aplysioviolin" in its ink-gland, which is derived from red algae consumed as part of its diet.

As regards *Cerithidea,* sources of pigments could also be attributed to nutritional factors. Examination of four species of algae which serve as food for the snail indicated the presence of chlorophylls and carotenoids, in addition to the phycobilins and chlorophyll *d* in the red alga. The snail probably builds up its pigment supply from these algal sources. β-Carotene seems to have been accumulated in the snail tissues without any metabolic alteration. However, the bluish-green color of mantle tissues is an indication that carotenoids may exist in them as a carotenoidprotein complex. The fact that no chlorophyll derivatives could be detected from these tissues also lends support to this conclusion. Zeaxanthin, carotenoid acids, and keto-carotenoids were not observed in the algae studied; they may be considered as products of metabolic activities of the snail. Moreover, some of the lutein and carotenoid acids were found esterified in the tissues of the snail. The spectral properties of chlorophyll derivatives indicate that these pigments must have also undergone some kind of metabolic change, possibly oxidation. The absorption maxima of the chlorophyll derivatives in the red region of the spectrum suggest that these pigments are derived from chlorophyll *a* of the algae. There was no indication of the presence of chlorophyll *d* or phycobilins in the tissues of the snail, negating the possibility that the snail absorbs these pigments from the red alga, *Hypnea*.

Several examples can be cited to prove that the accumulation of pigments in the body tissues of animals frequently results from catabolic activities. Such accumulation of pigments may or may not be significant in the functional economy of these organisms. It has been reported that the large pigment cells found in the deeper layer of connective tissues adjoining the intestinal caeca of the leech, *Glossiphonia complanata*, represent a kidney for the storage of waste products derived from haemoglobin metabolism (Bradbury, 1957). Wigglesworth (1943) found that in the blood-sucking bug, *Rhodnius prolixus*, some of the ingested blood is denatured to form biliverdin which is subsequently either excreted through the gut or stored in the pericardial cells. Stephenson (1947) noticed that the pigment in the gut epithelium of *Fasciola hepatica* is derived from the haemolysis of the ingested blood. The occurrence of chlorophyll derivatives in *Cerithidea* may not have any functional significance; they simply happen to be deposited in the tissues as metabolic wastes resulting from the digestion of algal food. Nevertheless, the storage of carotenoids in various tissues, particularly in the digestive gland of the snail, may be beneficial since there are indications that certain carotenoids may serve to prevent autoxidation of lipids in animal tissues (Verne, 1936a, 1936b). It is yet to be found out whether the snail needs vitamin A for its metabolic activities, and, in case it does, it might make use of β-carotene as a potential source. Although in certain invertebrates carotenoids are known to be utilized in sexual reproduction (Scheer, 1940) and in maintenance of mucous surfaces, nothing is known about their roles in similar processes in *Cerithidea*.

SUMMARY

1. Evidences obtained from chromatography, spectrophotometric absorption analyses, partition tests, etc. suggested the occurrence of the following pigments in the marine snail, *Cerithidea californica*: β-carotene, carotenoid acids, keto-carotenoids, lutein, and chlorophyll derivatives.

2. In an attempt to understand the dietary relationship of pigmentation in the snail, four species of algae were studied for their pigment contents. The three green algae were found to contain chlorophyll *a* and *b*, β-carotene, and lutein; the red alga, chlorophyll *a* and *d*, β-carotene, lutein, and phycobilins.

3. The spectral properties of the chlorophyll derivatives recovered from the snail suggested that they are derived from chlorophyll *a* of the algae and that the molecular structure is still intact with the magnesium atom atached to it. However,

absorption maxima in the violet region of the spectrum are shifted toward shorter, and in the red toward longer wave lengths, indicating some metabolic change in these pigments, possibly oxidation.

4. Part of the lutein and carotenoid acids were found to be esterified in the digestive gland and mantle tissues. No metabolic alteration has been noticed in the case of β-carotene. All available evidence suggests that zeaxanthin, carotenoid acids, and keto-carotenoids are products of the snail's metabolic activities.

5. Apparently the snails do not absorb phycobilins or chlorophyll *d* from the red alga.

6. The snail has been found to be non-selective in its chromatic storage.

7. The nutritional relationship and biological significance of pigments in the snail have been discussed.

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